

AN INVESTIGATION OF CRK PROTEIN KINASES OF *LEISHMANIA* AND THE ASSESSMENT OF THEIR POTENTIAL AS DRUG TARGETS

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Cyclin-dependent kinases (CDKs), exemplified by *cdc2*, are key regulators of the eukaryotic cell cycle [1]. A number of *cdc2*-related kinase genes have been isolated from trypanosomatids [2-6]. The present study was designed to identify and analyze *cdc2*-related protein kinases (CRKs), investigate their regulation mechanisms, and thus their role in regulating the cell cycle of the protozoa *Leishmania donovani*, the causative agent for Kala-azar and *Leishmania mexicana*; the causative agent for New world cutaneous leishmaniasis. To achieve this aim, CRK3 protein kinase gene was isolated from a Sudanese strain of *L. donovani*, designated *LdCRK3*. It encodes a protein of 311 amino acids with 99.7 % identity with the *L. mexicana* CRK3 and 49.4% identity with human Hscdc2. Southern blot analysis showed that *LdCRK3* is single copy, consistent with the genomic organization of all the so-far identified trypanosomatid protein kinases. The sequence of *L. donovani* CRK3 has been deposited in GenBankTM under the access number AJ426472.

Several lines of evidence have suggested that CRK3 is a potential drug target [7] thus chemical inhibitors could be tested against the enzyme. A second objective of this study was to provide an active protein kinase for that purpose. A system was developed to express and purify recombinant *L. mexicana* CRK1 & CRK3 proteins by Ni-NTA metal-chelate chromatography. CRK3 was over-expressed as a histidine fusion in *E. coli*. CRK3his purified from *E. coli* was found to be inactive towards histone H1 as a substrate, which is in contrast to CRK3his purified from transgenic *Leishmania*.

CDKs require a cyclin partner for their activation [1]. An analysis of data from the *L. major* sequencing project allowed the identification of a gene (*CYCX*) that encodes a protein (*CYCX*) with a cyclin fold motif. Oligonucleotides were designed to PCR amplify the cyclin gene from the *L. mexicana* genome. Southern analysis showed that this newly identified cyclin (*LmmCYCa*) is present as a single copy gene in the *L. mexicana* genome. *CYCa* showed 96% amino acid identity with the putative *L. major* cyclin and also a low, but significant, level of sequence identity to mitotic cyclins from other organisms.

The sequence of *L. mexicana* CYCa has been deposited in GenBank™ under the access number AJ426476. As previously shown [7], CRK3 is active at the G2/M phase of the *Leishmania* cell cycle; LmmCYCa is a good candidate to be the activating partner for CRK3. CYCa has been over-expressed in *E. coli* as a histidine fusion and purified by affinity chromatography. The protein is suitable for use in efforts to reconstitute active CRK3 *in vitro*.

The phosphorylation status of the recombinant CRK3his might be responsible for the lack of protein kinase activity. In order to check this hypothesis, Threonine-178 in CRK3his was changed to glutamic acid by site-directed mutagenesis of the expression plasmid, to generate the *L. mexicana* CRK3his-E178 mutant. The mutant protein kinase was found to be inactive, suggesting that phosphorylation status and cyclin binding are important for reconstituting protein kinase activity.

In parallel, the effect of Flavopiridol on the growth of the axenically grown amastigotes of *L. mexicana* was tested. It was found that the IC₅₀ for the amastigotes is 0.25µM, which is 10-fold less than that reported for the promastigotes [6], suggesting that growth inhibition is stage-specific and macrophage independent. Besides it provides base-line data for macrophage infectivity.

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